

## **II. REMARKS**

### **Status of the Claims**

Claims 1-72 remain pending in the case. Claim 50 has been amended to correct a clerical error and provide proper dependence. No new matter is added by these amendments, nor is it believed that any subject matter has been surrendered.

### **Information Disclosure Statement**

A copy of reference D6 of the IDS filed January 7, 2003 is enclosed. Copies of references D9 and D10 of the IDS filed December 15, 2003 will be forwarded under separate cover. Consideration of these references is requested.

### **Claim Rejections Under 35 U.S.C. § 102, second paragraph**

Claims 50-51 stand rejected under 35 U.S.C. § 102, second paragraph as being indefinite for lacking sufficient antecedent basis. Claim 50 has been amended to correct a clerical error and now properly recites "The vial of claim 35" thus overcoming this rejection. Claim 51 properly depends from Claim 50.

Claims 4-15 and 23-33 are rejected as being unclear as to whether the percentages are by weight or molar concentration. The applicant believes the Specification makes clear that all preservative concentrations are disclosed as weight/volume percentages. The Specification at page 14, lines 3-11 discusses various preservatives studied in Example 1, each described as a weight/volume measure, as, for example, "2-phenoxyethanol 0.5% w/v" and "benzethonium chloride 0.01% and 0.02% w/v." As the Specification makes clear that all preservative concentrations are weight/volume, no amendment of the claims is necessary.

### **Claim Rejections Under 35 U.S.C. § 102(b)**

Claims 20, 23-27, and 34 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Kakimoto et al. (EP 0 199 992). Applicant respectfully traverses this rejection.

A claim can only be anticipated if the cited reference teaches each and every element of the claim. Kakimoto allegedly discloses the use of benzethonium chloride to prevent adsorption of peptides, particularly those with molecular weights of 200 to 6000 (p. 5, lines 18-26), upon contact with various container materials. Kakimoto does not disclose the use of benzethonium chloride to inhibit microbial growth, nor does it discuss or disclose any use of benzethonium chloride beneficial to maintaining peptide viability over any extended period of time. Further, Kakimoto makes no reference to erythropoietin (which has a molecular weight of over 30,000) or any pharmaceutical preparations or compositions suitable for use with erythropoietin. As Kakimoto fails to teach each and every element of the present invention, Applicant respectfully requests that this rejection be withdrawn.

Claim 1 stands rejected under 35 U.S.C. § 102(b) as being anticipated by Nomura et al. (EP 0 459 795). Applicant respectfully traverses this rejection.

Nomura et al. allegedly discusses the use of benzethonium chloride as a surfactant for oral dose preparations of erythropoietin in amounts between 0.1 and 1000 parts by weight to one part per weight of erythropoietin. There is no mention of using benzethonium chloride as a preservative. Further, the reference does not discuss or provide any indication whether an amount of benzethonium chloride as disclosed for surfactant use would have any antimicrobial effect. In fact, since benzethonium chloride is not used in any of the specific examples, it remains unclear what concentration of benzethonium chloride would be necessary to function as an effective surfactant and whether such concentration would also be effective as an antimicrobial. As Nomura fails to teach each and every element of the present invention, Applicant respectfully requests that this rejection be withdrawn.

**Claim Rejections Under 35 U.S.C. § 103(a)**

Claims 1, 4, 5, 7, 8, 16-20, 23, 24, 26, 27, 34-35, 50-54, 57-61, and 69-72 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Strickland et al. (U.S. Patent No. 5,661,125) in view of Uda et al. (U.S. Patent No. 5,554,378) and Sandeep et al. (PDA J. Pharm. Sci. Technol. 1997 51(4): 166-71).

The Examiner has not established a *prima facie* case of obviousness. To maintain an obviousness rejection, the combined references must teach or suggest each and every claim limitation. Strickland has been discussed at length by the Applicant at p. 4, line 24 to p. 5, line 5 of the Specification where Applicant specifically points out that Strickland acknowledges difficulties associated with EPO formulations and Strickland explicitly states "...nothing specific can be derived from the use of preservatives with other proteins that would suggest any particular preserved formulation for erythropoietin." Further, while Strickland is allegedly directed to stable EPO formulations, there is absolutely no mention or suggestion that benzethonium chloride could be substituted for benzalkonium chloride or that use of benzethonium chloride would overcome the difficulties with EPO-compositions specifically highlighted by Strickland. Further, by mentioning benzalkonium chloride without ever discussing the possibility of using benzethonium chloride, Strickland teaches away from the very substitution suggested by the Examiner.

Uda does not cure this deficiency. As noted by both Strickland and the present Applicant, stable, sterile, multi-dose EPO formulations are few due in part to the particular instability of EPO and its tendency to interact with common pharmaceutical ingredients. Uda mentions EPO as one of several dozen potential peptides or proteins and mentions benzethonium chloride as a possible preservative that may be useful for intranasally-delivered peptide

compositions, but never identifies any particular combination of peptides with benzethonium chloride that would be effective. Further, neither erythropoietin nor benzethonium chloride are used in any of the specific examples and there is no teaching or suggestion that using benzethonium chloride with erythropoietin would result in the present compositions.

As the Examiner has failed to establish a prima facie case of obviousness, Applicant respectfully requests that this rejection be withdrawn.

Claims 2-3, 6, 9-15, 21-22, 25, 28-33, 36-49, 55-56, and 62-68 are rejected under 35 U.S.C. § 103(a) as unpatentable over Strickland et al., Uda et al., and Sandeep et al. in further view of Hyman et al. (U.S. Patent No. 3,389,837).

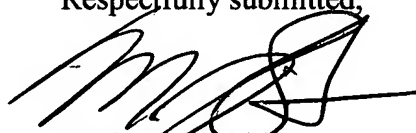
Strickland, Uda, and Sandeep have been discussed above and for the reasons stated, the Examiner has failed to establish a prima facie case of obviousness using those references. That deficiency is not cured by Hyman et al. which allegedly discloses that 9-aminoacridine hydrochloride and benzethonium chloride interact synergistically to inhibit *E. coli* growth. However, there is nothing in Hyman or any other cited reference to suggest that the synergistic effect of combining 9-aminoacridine hydrochloride with benzethonium chloride would be achieved with any other combination of preservatives. Further, there is nothing in Hyman to suggest that such a combination could be used to create pharmaceutical compositions, particularly compositions requiring maintenance of protein stability. Hyman supposedly discloses an antibacterial soap or detergent and contains no suggestion to any possible pharmaceutical use.

As the Examiner has failed to establish a prima facie case of obviousness, Applicant respectfully requests that this rejection be withdrawn.

**Conclusion**

Applicant submits that, based on the Amendments and Remarks herein, the claims are in condition for allowance and such favorable action is respectfully requested. If the Examiner has any questions or comments that might accelerate allowance of these claims, he is invited to contact the undersigned representative at (512) 542-8530.

Respectfully submitted,



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CERTIFICATE OF MAILING

I, certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to the Commissioner for Patents, U.S. Patent and Trademark Office, P. O. Box 1450, Alexandria, VA 22313-1450 on the date below:

September 27, 2004  
Date

Michael J. Smith

Commissioner for Patents  
U.S. Patent and Trademark Office  
P. O. Box 1450  
Alexandria, VA 22313-1450

Re: U.S. Patent Application Serial No. 10/031,947 entitled "Multi-dose Erythropoietin Formulations," by Atef Gayed  
(Our Ref: MAR618/4-005(A)US/75000)

Sir:

Enclosed for filing in the above-referenced patent application is:

1. Amendment and Response to Office Action mailed May 26, 2004;
2. A credit card authorization for \$110; and
3. A return postcard to acknowledge receipt of these documents. Please date stamp and mail this postcard.

Should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, the Assistant Commissioner is hereby authorized to deduct said fees from VINSON & ELKINS L.L.P. Deposit Account No. 22-0365/MAR618/4-005(A)US/75000.

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102061:3058  
Enclosures

## Purification of Human Erythropoietin\*

(Received for publication, January 24, 1977, and in revised form, April 11, 1977)

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Human erythropoietin, derived from urine of patients with aplastic anemia, has been purified to apparent homogeneity. The seven-step procedure, which included ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, yielded a preparation with a potency of 70,400 units/mg of protein in 21% yield. This represents a purification factor of 930. The purified hormone has a single electrophoretic component in polyacrylamide gels at pH 9, in the presence of sodium dodecyl sulfate at pH 7, and in the presence of Triton X-100 at pH 6. Two fractions of the same potency and molecular size, by sodium dodecyl sulfate gel electrophoresis, but differing slightly in mobility at pH 9, were obtained at the last step of fractionation. The nature of the difference between these two components is not yet understood.

Erythropoietin is an acidic glycoprotein that is present at a very low concentration in plasma under normal conditions. Under anemic or anoxic stress, it is found in relatively large amount in the plasma and is also excreted in the urine. Erythropoietin is the substance that is responsible, in large part, for the regulation of normal red blood cell differentiation. Because of this function, and because it may have a role in replacement therapy of some kinds of anemia, it is important to have pure erythropoietin in an amount sufficient for chemical characterization. Reports on the purification of human (1) and sheep (2) erythropoietin have been published. In the former, the evidence for homogeneity was not convincing, and in the latter, the total amount was too low for adequate characterization. We report in this paper on the preparation of milligram quantities of human urinary erythropoietin in a state of apparent homogeneity.

### EXPERIMENTAL PROCEDURES

**Bioassay**—The fasted rat method of bioassay (3), in which the incorporation of labeled iron into circulating red cells is measured, was used routinely to quantitate the amount of erythropoietin activity. Samples for assay were dissolved in 0.1% bovine serum albumin in 0.15 M NaCl, 0.01 M CaCl<sub>2</sub>. Over the 18-month period covered by this work, the  $\ln$  dose- $\ln$  response curve obtained when 1, 1.5, 2, and

3 units of erythropoietin<sup>1</sup>/rat were used had the following characteristics: slope,  $1.11 \pm 0.34$ ; intercept,  $0.75 \pm 0.39$ ; correlation coefficient,  $0.96 \pm 0.10$ . The assay values found for the two final hydroxylapatite fractions were confirmed by the polycythemic mouse method (3) which agreed closely with the other two assay methods. We are indebted to Dr. Walter Fried of the Michael Reese Hospital for doing the mouse assays. For the iodinated preparation and for the assay of activity recovered from polyacrylamide gels, biological activity was measured by the marrow cell culture method (4). This procedure, in which both the total uptake of radio-iron and its incorporation into hemoglobin are used as quantitative indicators of erythropoietin activity, is about 1000 times more sensitive than the fasted rat method, but does not distinguish between native erythropoietin and the asialo form, which is inactive *in vivo*.

**Materials**—Sodium dodecyl sulfate and DEAE-agarose were bought from Bio-Rad Laboratories, Richmond, Calif., as was hydroxylapatite (Bio-Gel HT, Control 12746); we found no significant difference between several different lots which we used. Sulfopropyl Sephadex (Lot 7963) and Sephadex G-100 (Lot 5011) were bought from Pharmacia Inc., Piscataway, N. J. Materials for gel electrophoresis (acrylamide, *N,N,N',N'*-tetramethylethylenediamine and *N,N'*-methylenebisacrylamide) and Triton X-100, scintillation grade, were bought from Eastman Kodak Co., Rochester, N. Y. Labeled iodide was obtained from Amersham-Searle Corp., Arlington Heights, Ill. Other reagents used were of the best quality commercially available. Ultrafilters were bought from Amicon Corp., Lexington, Mass. PBS is used to designate a solution consisting of 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0.

**Iodination**—Labeling with <sup>125</sup>I (5, 6) was done as follows. To 20  $\mu$ l of an erythropoietin solution containing 20  $\mu$ g of protein, 2  $\mu$ l of 0.5 M phosphate, pH 7.0, and 20  $\mu$ l of dimethylsulfoxide were added. One microliter of Na<sup>125</sup>I (100  $\mu$ Ci, equivalent to 7.14 ng of iodide or 57 pg atoms) was then added, followed by 1  $\mu$ l of freshly prepared chloramine-T (10 mg/ml in water). The mixture was allowed to stand at 24° for 10 min, after which 10  $\mu$ l of Na<sub>2</sub>S<sub>2</sub>O<sub>6</sub> (25 mg/ml in water) were added. The solution was mixed and allowed to stand for 1 min; then 200  $\mu$ l of KI (10 mg/ml in 0.05 M phosphate, pH 7.4) were added and mixed for 1 min at 24°, followed by addition of 50  $\mu$ l of 7% (w/w) bovine serum albumin. The mixture was put on a Sephadex G-10 column (25  $\times$  0.9 cm diameter), which had been equilibrated with PBS, being washed over to the column with two 200- $\mu$ l washes of KI solution (10 mg/ml). The erythropoietin was separated from unreacted iodide by elution with PBS and collection of 0.3-ml fractions. The major peak material of large molecular weight label (Tubes 18 to 28) was pooled and dialyzed. The final volume of 4.1 ml contained  $5.8 \times 10^7$  cpm of <sup>125</sup>I ( $2.9 \times 10^6$  cpm/ $\mu$ g of protein, equivalent to 0.1 g atom of iodine/mol of protein).

Because our previous experience showed that sheep erythropoietin was completely inactivated upon iodination using chloramine-T, we used the method of Stagg *et al.* (5), in which the presence of

\* This work was supported in part by United States Public Health Service Grant HL 16005-03.

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§ Operated by the University of Chicago for the United States Energy Research and Development Administration under contract EY-76-C-02-0069.

<sup>1</sup> One unit of erythropoietin is defined as the biological activity present in one-tenth of the contents of an ampule of the International Reference Preparation distributed by the World Health Organization. In the routine assay, we used, as a working standard, a preparation of sheep erythropoietin that had been standardized against the International Reference Preparation.

dimethylsulfoxide acts to protect methionine residues from oxidation. At the level of 0.1 atom of iodine/molecule, we found no loss of biological activity when the assay was done within 1 day of iodination. One week later, however, there was appreciable loss (36%), indicating that the procedure had labilized the hormone. At a higher degree of iodination (4 atoms/molecule), 75% of the biological activity was lost within 1 day.

**Electrophoresis**—Polyacrylamide gel electrophoresis was done by the micromethod which we had used earlier (2), with gels that were 5 × 0.2 cm in diameter. The conditions are given below for each experiment. Gels were fixed in 25% isopropyl alcohol, 10% acetic acid overnight, stained in 0.25% Coomassie blue in 10% acetic acid for 1 h, and destained in 10% acetic acid.

**Source of Erythropoietin**—Urine, 2550 liters, was collected from two groups of patients with aplastic anemia of unknown origin, in several hospitals in Kumamoto City, Japan. These groups included some patients with moderately severe, chronic anemia for whom the urine titer was about 1 unit/ml, and others with severe, chronic anemia for whom the urine titer was 2 to 6 units/ml. The urine was collected in 11 pools and filtered under suction; 2.5-liter batches were deionized on a Sephadex G-50 column (57 × 15 cm diameter; bed volume, 10 liters). The effluent (3.5 liters) was made 0.029 M with respect to both  $\text{NaH}_2\text{PO}_4$  and  $\text{NaCl}$ , and 2.5 g (dry weight) of DEAE-cellulose, previously equilibrated with 0.025 M  $\text{NaH}_2\text{PO}_4$ , were stirred into the solution. After 30 min of stirring at 4°, the DEAE-cellulose was allowed to settle for 2 h in the cold and then collected on a sintered glass filter with the aid of gentle suction. The adsorbed activity was immediately eluted four times with 25 ml of 0.05 M  $\text{Na}_2\text{HPO}_4$ , 0.15 M  $\text{NaCl}$ . The eluate was dialyzed against deionized water (two changes of 2 liters each) overnight and lyophilized. The total yield of this fraction was 6.976 million units of activity with a mean potency of about 90 u/A.<sup>3</sup> Previous experience had shown that the desalting step on Sephadex G-50 had a yield of 80%, and that the adsorption and elution from DEAE-cellulose yielded 90% of the input activity (7).

## RESULTS

All of the urine concentrates were treated with phenol p-aminosalicylate, as described by Chiba *et al.* (8), so that the loss of activity due to enzymic degradation was reduced. This procedure was carried out on 18 batches which consisted of a total of 7,059,670 units<sup>3</sup> and a mean potency of 91 u/A (range, 16 to 160). There were 5,115,110 units recovered, with a mean potency of 109 u/A. In spite of the fact that 28% of the activity was lost and the mean purification factor was only 1.20, it was necessary to use this technique to avoid major losses later in the purification process.

The purification method described below was developed as a result of many trials of various standard techniques. For example, we found that use of gel permeation chromatography early in the procedure did not lead to any significant purification, probably due to the large amount of glycoprotein with similar sizes in the crude urine concentrate; stepwise elution of ion exchange columns was used throughout the procedure since we found that gradient elution decreased the resolution.

**Ethanol Precipitation**—Sixteen separate batches were precipitated with ethanol by the following procedure. The sample, e.g. 111,600 units at 52 u/A, was dissolved in 50 ml of PBS at 4°; 5  $\mu$ l were removed for assay, and 12.5 ml of 10 M LiCl<sup>4</sup> were added. Absolute ethanol (62.5 ml) at 4° was added slowly with stirring, which was continued for 30 min after the addition

was complete. After the flocculent precipitate had been allowed to settle for 15 min, it was removed by centrifugation at 21,000 × *g* for 10 min at -15°. The pellet was washed three times with 10 ml of 50% ethanol, 1 M LiCl and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS, yielding a turbid solution (50% precipitate).

Sixty-seven milliliters of absolute ethanol were added slowly to the combined supernatants; stirring was continued for 30 min and settling for 15 min. The precipitate was collected as before and washed twice with 10 ml of 65% ethanol, 0.7 M LiCl, and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS (65% precipitate).

To the pooled supernatants, 96 ml of ethanol were added slowly, and stirring was continued for 30 min, after which the precipitate was allowed to settle for 14 h at 4°. We have found that this long period in 75% alcohol is required for optimal further fractionation. The precipitate was washed twice with 10 ml of 75% ethanol, 0.5 M LiCl, the supernatants were pooled, and the precipitate was dissolved in 20 ml of PBS (75% precipitate).

The combined supernatant was brought to 90% ethanol by addition of 540 ml of absolute alcohol, stirred for 30 min, and stored at -20° for 48 h before the precipitate was collected, dissolved in 50 ml of cold water, and immediately frozen (90% precipitate). The results of one representative ethanol fractionation procedure are given in Table I.

For the 16 experiments, the range of yields in the 90% ethanol precipitate was 28 to 100%. The range of potency was 133 to 880 u/A.

Since a substantial fraction of the activity was found in the earlier alcohol precipitates, we established conditions for recovery of much of that activity at a potency similar to that of the 90% ethanol precipitate. For example, three groups of pooled fractions (50%, 65%, and 75% alcohol precipitates), with a total volume of 210 ml, were stirred at 4° while 120.37 g of guanidine hydrochloride were added. To the clear solution, 52.5 ml of 10 M LiCl were added with continued stirring. The slightly cloudy solution was stirred for 30 min more, and 790 ml of absolute ethanol was added slowly. After 30 min of stirring, the suspension was kept at 4° for 25 h, and the precipitate was collected by centrifugation at 21,000 × *g* for 15 min at -15°, to yield Supernatant A and a pellet. The pellet was suspended in 50 ml of PBS and stirred for 30 min while 28.66 g of guanidine hydrochloride were added. During the next 20 min, 12.5 ml of 10 M LiCl were added, followed by 187.5 ml of absolute ethanol. Stirring was continued for 30 min, after which the suspension was allowed to settle at 4° for about 14 h. The precipitate was removed at 21,000 × *g* for 15 min, yielding another 75% ethanol supernatant. The re-extraction was repeated; the two supernatant fractions (Supernatant B) were pooled, but this pool was kept separate from the original Supernatant A. The 75% alcohol precipitate was suspended in 200 ml of PBS and mixed thoroughly.

To Supernatant A, 1052 ml of absolute ethanol were added

TABLE I  
Ethanol fractionation

Fraction	A	Activity u	u/A	% yield	Purification
Original	2,150	111,600	52	100	
50% precipitate	570	8,900	16	8	
65% precipitate	306	16,100	53	14	1.02
75% precipitate	131	16,800	129	15	2.5
90% precipitate	173	76,800	443	69	8.5

<sup>3</sup> Potency, or specific activity, is expressed as units of biological activity (u) per absorbance unit (A), measured at 278 nm in 1-cm cuvettes.

<sup>4</sup> This figure is slightly different (1.2% higher) from that indicated as the amount obtained from the DEAE-cellulose step. This kind of difference is caused by the uncertainty in the bioassay and will also be seen at subsequent steps.

<sup>5</sup> LiCl was used in the alcohol precipitation procedure in order to increase the solubility of proteins in ethanol (9). Precipitation in the absence of salt resulted in a low potency fraction.



slowly; the suspension was stirred for an additional 30 min and stored at  $-20^{\circ}$  for 40 h. The precipitate was then collected at  $21,000 \times g$  for 15 min at  $-15^{\circ}$ , washed twice with 40 ml of 90% ethanol, 0.2 M LiCl, and suspended in 30 ml of PBS. After stirring for 30 min, the suspension was centrifuged at  $16,000 \times g$  for 10 min at  $4^{\circ}$ ; the small amount of precipitate was washed with 20 ml of PBS, and the supernatant solutions were pooled (90% ethanol precipitate). The pellet fraction was suspended in 20 ml of PBS. Supernatant B was treated in an analogous manner, i.e. the 90% alcohol precipitate was collected and both Supernatants A and B were assayed. The fraction precipitated at 90% alcohol from Supernatant A contained 246,840 units (54% of the input activity) with a potency of 560 u/A. The fraction precipitate from Supernatant B contained 69,300 units (15% yield) with a potency of 565 u/A. When all of the available ethanol precipitates were re-extracted, we recovered 1,515,200 units with a mean potency of 570 u/A (range, 220 to 680). This material was pooled with the original 90% alcohol precipitates for further fractionation, making a total of 4,750,740 units at a mean potency of 633 u/A.

**DEAE-Agarose Fractionation**—The solution, in water, of a 90% ethanol precipitate was concentrated to about 5 ml on an Amicon UM-10 ultrafilter, then brought to 25 ml with 0.01 M Tris, pH 7.0, and a 50- $\mu$ l aliquot was removed. The DEAE-agarose, 100 to 200 mesh, was degassed under reduced pressure, suspended in 0.01 M Tris, pH 7.0, and packed into a column  $9.2 \times 2.5$  cm in diameter (bed volume, 45 ml). The gel was washed with 1.5 liters of 0.01 M Tris, pH 6.9; the ratio of absorbance units added to bed volume (ml) was 6.65. The sample was added to the column over a period of 40 min, and 150-drop fractions were collected. The column was washed with 211 ml of 0.01 M Tris, pH 7, and then eluted with the following buffers: 366 ml of 0.01 M Tris, pH 7.0; 5 mM  $\text{CaCl}_2$ ; 270 ml of 0.01 M Tris, pH 7.0; 17 mM  $\text{CaCl}_2$ ; 194 ml of 0.01 M Tris, pH 7.0; 30 mM  $\text{CaCl}_2$ ; and 65 ml of 0.1 M  $\text{CaCl}_2$ . The elution pattern can be seen in Fig. 1, and the results are given in Table II.

Of the 4,566,240 units of total input, we recovered 4,052,710 (89%) in the 17 mM  $\text{CaCl}_2$  eluate at a mean potency of 1,110 u/A, representing a mean purification factor of 1.97. From this point on in the fractionation calcium was added to all buffers except those used with hydroxylapatite columns because there were inconsistent results and appreciable losses of activity

when buffers without calcium were used. For the next step in purification, we selected three eluates from DEAE-agarose columns, amounting to 2,480,400 units (61% of the total yield) with a mean potency of 1,750 u/A.

**Sulfopropyl-Sephadex Chromatograph**—The three eluates (17 mM  $\text{CaCl}_2$ ) from DEAE-agarose columns were desalted and concentrated on a UM-10 ultrafilter and then dialyzed against 2 liters of 5 mM  $\text{CaCl}_2$ , pH 7.5, overnight. In the sample run described below, 30 ml of dialyzed solution were brought to pH 4.50 by dropwise addition of 0.1 M HCl; the small amount of precipitate formed was removed by centrifugation and washed with 5 ml of 5 mM  $\text{CaCl}_2$ , pH 4.5. The wash, pooled with the supernatant, was applied to a sulfopropyl-Sephadex column ( $15.0 \times 2.5$  cm in diameter; bed volume, 78.3 ml) which had been equilibrated with 5 mM  $\text{CaCl}_2$ , pH 4.50. The absorbance units to bed volume (ml) ratio was 2.47. We found that a low value for this ratio is critical for optimal fractionation on sulfopropyl-Sephadex; for example, if the absorbance unit to bed volume ratio was greater than 10, almost all of the activity was found in the effluent fraction. The following buffers were used in developing the column. Input was: 5 mM calcium acetate, pH 4.50, specific conductivity =  $1,075 \mu\text{mho cm}^{-1}$ . Eluting buffers were: 7.5 mM calcium acetate, pH 4.70, specific conductivity  $1,500 \mu\text{mho cm}^{-1}$ ; 12.5 mM calcium acetate, pH 5.25, specific conductivity =  $2,100 \mu\text{mho cm}^{-1}$ ; 15 mM calcium acetate, pH 5.5, specific conductivity =  $2,400 \mu\text{mho cm}^{-1}$ ; 0.1 M calcium acetate, 0.01 M Tris, pH 7.24, specific conductivity =  $11,500 \mu\text{mho cm}^{-1}$ . The column was run at 0.4 ml/min at  $4^{\circ}$ , and 200-drop fractions were collected. After a reading was taken at 278 nm and the appropriate pools were made, the solutions were neutralized (within 1 h after elution), and aliquots were removed for assay and stored at  $-20^{\circ}$ . The elution pattern is presented in Fig. 2 and results of the fractionation are shown in Table III.

The overall results of this step in the purification were: 55% recovery (1,352,810 units) in the 12.5 mM calcium acetate, pH 5.55 fraction, at a mean potency of 11,170 u/A, and with a mean purification factor of 6.38.

**Gel Filtration**—The 12.5 and 15 mM calcium acetate eluates from the sulfopropyl-Sephadex column separations were run in two separate batches on the same gel column. The pools were concentrated on Amicon UM-2 ultrafilters to about 5 ml and equilibrated with 10 mM  $\text{CaCl}_2$ , 10 mM Tris, pH 6.87, before application to the column. The Sephadex G-100 gel was degassed under reduced pressure and equilibrated with the same buffer before the column was poured. The column ( $100 \times 2.5$  cm diameter) was calibrated with markers of known molecular size before being used for the erythropoietin fractions. The void volume was 135 ml; bovine serum albumin monomer eluted at 224 ml, ovalbumin at 258 ml, and cytochrome c at 368 ml. The sample was added to the bottom of the column, as was the buffer which was passed through the column at 21 to 22 ml/

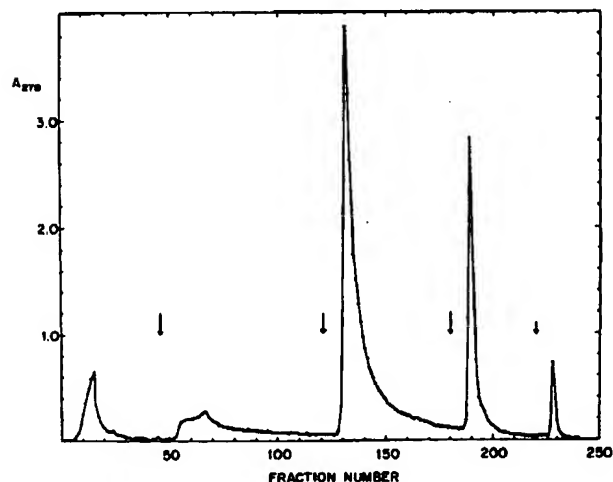


FIG. 1. DEAE-agarose chromatography of erythropoietin. Buffer changes are indicated by arrows as specified in the text.

TABLE II  
DEAE-agarose fractionation

Fraction	A	u	u/A	% recovery of activity	Purification factor
Input	299	164,030	549	100	
0.01 M Tris	23	51	2		
0.01 M Tris, 5 mM $\text{CaCl}_2$	37	9	0.2		
0.01 M Tris, 17 mM $\text{CaCl}_2$	158	143,240	907	87	1.65
0.01 M Tris, 30 mM $\text{CaCl}_2$	57	38,080	633	22	1.15
0.1 M $\text{CaCl}_2$	8	98	12		

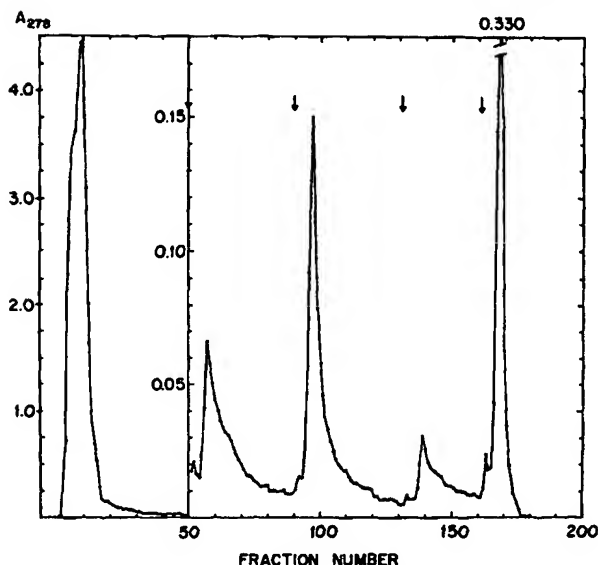


FIG. 2. Sulfopropyl-Sephadex chromatography of erythropoietin. Buffer changes are indicated by arrows as specified in the text.

TABLE III  
Sulfopropyl-Sephadex fractionation

Fraction	A	u	u/A	% recov- ery of ac- tivity	Purifica- tion factor
Input	193	198,160	730	100	
5 mM calcium acetate	138	5,610	27	3	
7.5 mM calcium acetate	8	16,920	3,170	9	4.3
12.5 mM calcium acetate	12	71,240	9,420	36	12.9
15 mM calcium acetate	9	18,600	6,360	9	8.7
0.1 M calcium acetate	19	10,810	3,040	5	4.1

h by means of a Mariotte bottle with a 42-cm hydrostatic head. Each fraction collected was 4.1 ml (120 drops), and the following pools were made: I, 0 to 131.2 ml; II, 131.2 to 184.5 ml; III, 184.5 to 205 ml; IV, 205 to 258.3 ml; and V, 258.3 to 328 ml (Fig. 3). The first four pools were concentrated by ultrafiltration and aliquots were assayed. In one of the runs, pools I and II contained 17% and 5% of the absorbance units, respectively, but no detectable activity; pool III contained 32% of the absorbance units and 104% of the input activity, yielding a fraction with a potency of 38,850 u/A; and pool IV contained 10% of the absorbance units and 2% of the biological activity. Pool V was not assayed.

For the combined two gel filtration runs, the yield in pool III (184.5 to 205 ml) was 100%, the mean potency was 39,060 u/A, and the purification factor was 3.04.

**Hydroxylapatite Chromatography**—Hydroxylapatite was packed under unit gravity into a column (6.1 × 1.5 cm diameter) and washed with 500 ml of water and then with 400 ml of 0.5 mM phosphate buffer, pH 7.1, conductivity = 69  $\mu\text{mho cm}^{-1}$  (Buffer I), by use of a peristaltic pump which maintained the flow at 0.3 ml/min. After the buffer wash, the length of the column was 3.4 cm and the bed volume was 6.0 ml. The input sample was concentrated and desalted on an Amicon DM-5 ultrafilter by adding water to the concentrate and reconcentrating three times. The final concentrate and the wash of the filter were centrifuged at  $6,000 \times g$  for 20 min at 4°. The small insoluble pellet was washed once with 0.5 mM phosphate, pH 7.1, and the wash was added to the supernatant. An aliquot for assay was removed and the remainder (22 ml) was added to

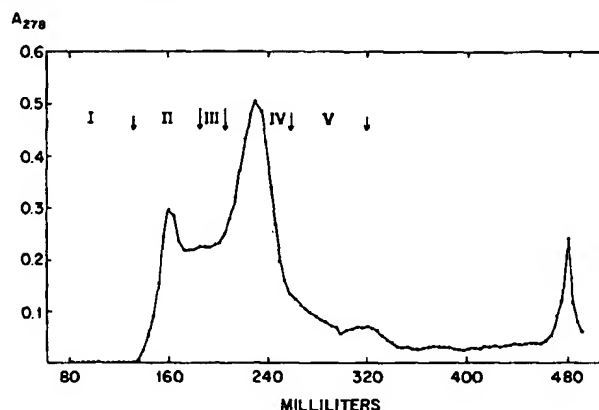


FIG. 3. Gel filtration chromatography of erythropoietin. The arrows indicate pools made for assay.

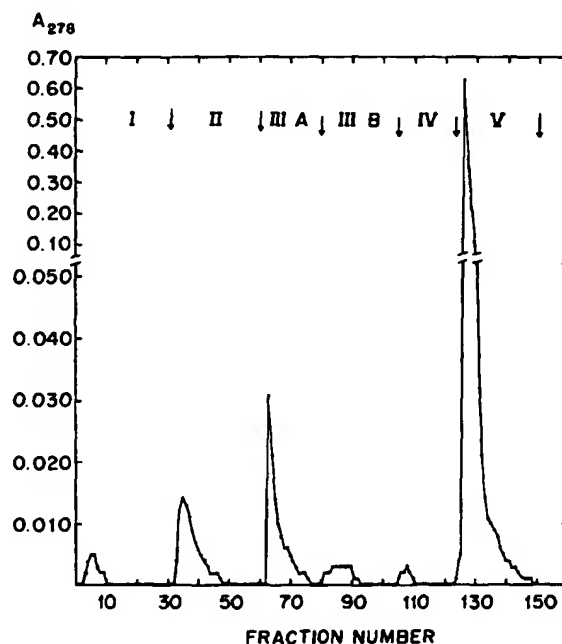


FIG. 4. Hydroxylapatite chromatography of erythropoietin. The arrows and Roman numerals indicate buffer changes and pools.

TABLE IV  
Hydroxylapatite chromatography of erythropoietin

	A	u	u/A	% recov- ery of ac- tivity	Purifica- tion factor
Input	11.0	381,480	34,680	100	
Effluent	0.35	490	1,400		
Fraction II	0.96	123,480	128,620	32	3.71
Fraction IIIA	0.85	79,850	93,940	21	2.71
Fraction IIIB	0.32	22,800	71,250	6	2.03
Fraction IV	0.29	6,260	21,590	2	
Fraction V	7.22	17,680	2,450	5	

the column. The ratio of absorbance units added to bed volume (ml) was 1.82. The input buffer was pumped through the column until the effluent  $A_{278}$  was less than 0.005 (149 ml), and the following elution schedule was carried out: Buffer II, 1 mM phosphate (pH 7.1, specific conductivity 131 =  $\mu\text{mho cm}^{-1}$ , 150 ml (Fraction II)); Buffer III, 2 mM phosphate (pH 6.9, specific conductivity = 270  $\mu\text{mho cm}^{-1}$ , 220 ml (Fractions IIIA and

TABLE V

Purification of erythropoietin: summary

After the DEAE-agarose step, only 61% of the product was used in further fractionation.

Step	Input		Product		Yield (%)		Mean purification factor
	u	Potency u/A	u	Potency u/A	Each step	Overall	
DEAE-cellulose			6,876,170	89	100	100	
Phenol	7,059,670	91	5,115,110	110	72	72	1.21
Ethanol	5,186,690	88	4,750,740	660	92	66	7.50
DEAE-agarose	4,566,240	563	4,052,710	1,107	89	59	1.97
Sulfoethyl-Sephadex	2,480,400	1,750	1,352,810	11,170	55	32	6.38
Sephadex G-100	1,259,040	12,830	1,274,430	39,060	100	32	3.04
Hydroxylapatite	1,083,650	38,770	721,160	82,720	67	21	2.13

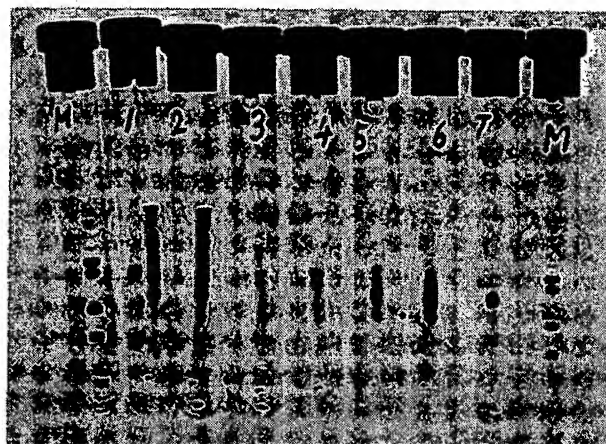


FIG. 5. SDS-polyacrylamide electrophoretic analysis of the most active fractions from each step in the purification of human erythropoietin. The gels marked M had serum as marker proteins. 1, DEAE-cellulose eluate; 2, phenol-treated; 3, 90% ethanol precipitate; 4, DEAE-agarose (17 mM  $\text{Ca}^{++}$ ) eluate; 5, sulfoethyl-Sephadex (12.5 mM  $\text{Ca}^{++}$ ) eluate; 6, Sephadex G-100 (pool III); 7, hydroxylapatite Fraction II. The gel concentration was 7.5%.

IIIB); Buffer IV, 3 mM phosphate (pH 6.9, specific conductivity = 402  $\mu\text{mho cm}^{-1}$ , 84 ml (Fraction IV)); Buffer V, 0.1 M phosphate (pH 6.8, specific conductivity = 9.6  $\text{mmho cm}^{-1}$ , 134 ml (Fraction V)). The elution pattern is shown in Fig. 4; the results for one such column are listed in Table IV.

The total input for the two runs was 1,083,650 units, with a mean potency of 38,770 u/A. The total recovered in Fractions II and IIIA was 721,163 units (67%) with a mean potency of 82,720 u/A and a mean purification factor of 2.13. Each of the fractions, II and IIIA, from the two experiments was concentrated by means of Amicon DM-5 ultrafilter and stored frozen.

When we examined Fractions II and IIIA from the two hydroxylapatite columns by gel electrophoresis in SDS<sup>a</sup> (7.5% gels), we found single bands, each with a relative mobility (with reference to the Pyronin Y band) of 0.50. No detectable difference in mobility, in the presence of SDS, between Fractions II and IIIA could be found. Fig. 5 shows the SDS-gel electrophoretic analysis of each of the most active fractions throughout the purification procedure, and Table V summarizes the seven-step method. The overall purification factor was 929 (calculated from initial and final potencies).

Since the hydroxylapatite Fractions II and IIIA appeared to have a single, identical component on SDS gels, we examined

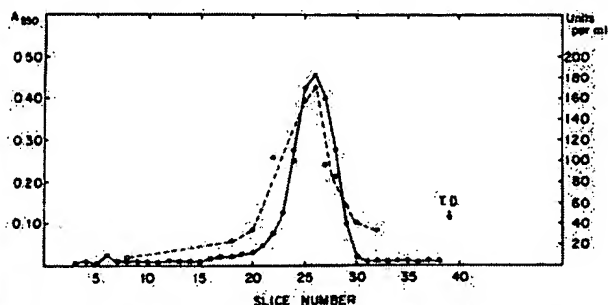


FIG. 6. Gel electrophoresis (pH 9) of erythropoietin (hydroxylapatite Fraction II). ● represents absorbance of the Coomassie blue-stained material, and ○ represents biological activity. The gel concentration was 8%. T. D., tracking dye.

these fractions further for evidence of heterogeneity. When these fractions were compared by gel electrophoresis at pH 9, it was clear that there was a small, but significant difference in mobility. Fraction II had a mobility relative to the bromophenol blue tracking dye of 0.49, and the value for Fraction IIIA was 0.52. In spite of our finding of similar potency and molecular size, these two preparations must be considered different. The chemical basis for this difference is now being studied.

Fraction II was run on two gels at pH 9; one was fixed, stained, and scanned, and the other was sliced into 1.1-mm slices which were put into 0.5 ml of 0.10% bovine serum albumin, 10 mM  $\text{CaCl}_2$  in 0.15 M NaCl, and the hormone was allowed to diffuse out of the gel at 4° for 18 h. On assay by the *in vitro* method, we found the biological activity coincident with the single band of stained protein (Fig. 6).

In view of our previous finding that native sheep erythropoietin was very poorly fixed to polyacrylamide gels and was largely lost during the staining procedure, we adopted the expedient used earlier for the sheep hormone. Fraction II was iodinated with <sup>125</sup>I, run on a gel at pH 9 which was then cut into 1.1-mm slices, and counted before and after fixation. The results in Fig. 7 show a single peak of labeled hormone, only a fraction (44%) of which was fixed. The iodinated hormone was then run on a gel at pH 6 in order to confirm the apparent homogeneity. It became clear that there was a large degree of aggregation at the lower pH, since only a small amount of the radiolabeled hormone could be found in the gel, with the major fraction remaining at the origin. We then used the observation of Kawasaki and Ashwell (10), who found that aggregation of a liver glycoprotein could be reduced by the use of Triton X-100. When both the native and asialo forms of erythropoietin were run on gels in the presence of 0.05% Triton X-100 (Fig. 8), we found for the former a single symmetrical peak and for the

<sup>a</sup> The abbreviation used is: SDS, sodium dodecyl sulfate.

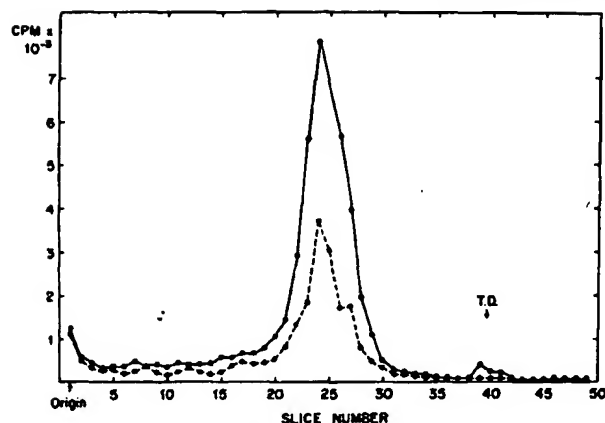


FIG. 7. Gel electrophoresis (pH 9) of  $^{125}\text{I}$ -labeled erythropoietin (hydroxylapatite fraction II).  $\bullet$ — $\bullet$  represents total radioactivity;  $\circ$ — $\circ$  represents radioactivity remaining in the gel slices after fixation. The gel concentration was 8%. T. D., tracking dye.

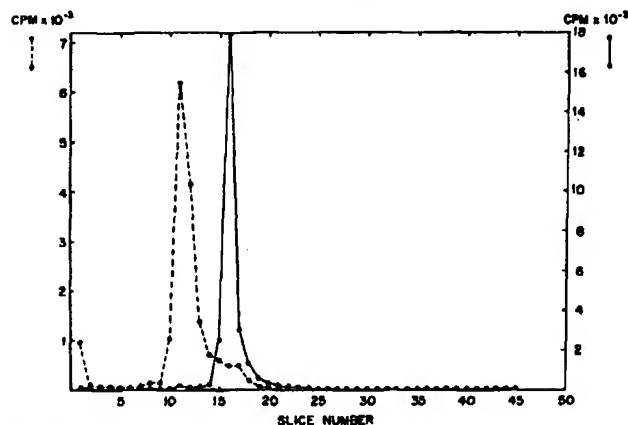


FIG. 8. SDS-gel electrophoresis of  $^{125}\text{I}$ -labeled erythropoietin (hydroxylapatite Fraction II).  $\bullet$ — $\bullet$  represents native erythropoietin;  $\circ$ — $\circ$  represents asialoerythropoietin. The gel concentration was 10%.

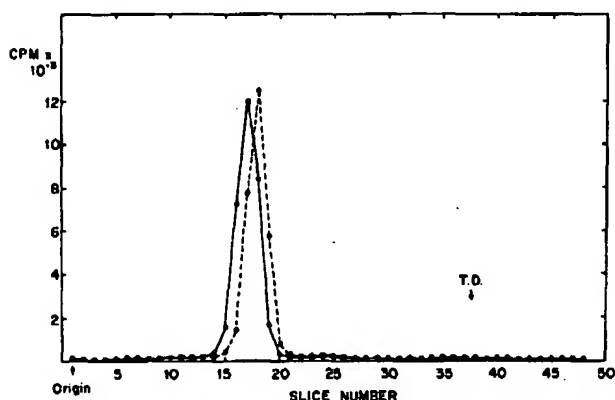


FIG. 9. Gel electrophoresis (pH 6, 0.05% Triton X-100) of  $^{125}\text{I}$ -labeled erythropoietin (hydroxylapatite Fraction II).  $\bullet$ — $\bullet$  represents native erythropoietin;  $\circ$ — $\circ$  represents asialoerythropoietin. The gel concentration was 10%. T. D., tracking dye.

latter, one major symmetrical peak with only a trace of the native hormone contaminating the asialo form. At a lower concentration of Triton X-100 (0.01%), there was still appreciable aggregation, as detected by label that remained at the origin.

Examination of iodinated Fraction II, both native and asialo, on SDS gels (11) showed single, symmetrical peaks (Fig. 9) with no evidence of heterogeneity with respect to size.

We measured the absorbance at 278 nm and at 191 nm, using crystalline bovine serum albumin as a standard and correcting for stray light at 191 nm, and found that  $A_{191}^{278}$  for erythropoietin is 8.51. Using this value, the mean potency of homogeneous human erythropoietin can be expressed as 70,400 units/mg of protein.

#### DISCUSSION

Espada and Gutnisky (1) isolated a fraction, from urine of patients with anemia due to hookworm, that had a potency of about 8,000 units/mg of protein. They claimed, on the basis of a gel permeation experiment, that this fraction was homogeneous; the poor resolution characteristic of this method of analysis, however, makes it necessary to use additional kinds of information to establish purity. In a subsequent paper, Espada *et al.* (12) claimed that the same preparation was homogeneous by gel electrophoresis at pH 9, although they pointed out that the stained band was diffuse. In addition, these authors showed an immunodiffusion pattern that was inconclusive with respect to immunological homogeneity. Our finding that human erythropoietin has a minimal potency of 70,400 units/mg of protein suggests that Espada's preparation is either about 11% pure or, if it is homogeneous, is largely in the asialo form that has no activity *in vivo*.

Our previously reported data (2) indicated that the preparation of sheep plasma erythropoietin, with a potency of 9,200 u/A, was free of any contaminant except for a small amount of asialoerythropoietin. If this is truly the case, then human urinary erythropoietin is 7 to 8 times more active than the sheep hormone, when assayed by the same method. This may be due to a greater sensitivity of rats to the human than to the sheep hormone, or it may indicate that human urinary erythropoietin is intrinsically more active than sheep plasma erythropoietin.

The appearance of two fractions with the same potency, as a result of hydroxylapatite fractionation, suggests a degree of heterogeneity which is not detected upon electrophoresis in SDS, and which might be accounted for by a small difference in the number of terminal sialic acids or of amide groups, or of both. Our findings of single peaks upon electrophoresis at pH 9, pH 6, and pH 7 in SDS constitute reasonable evidence of homogeneity with respect to charge and molecular size for each of the two fractions. At pH 6 in the presence of Triton X-100, the native and asialo forms are clearly separated (Fig. 9), and we could expect to be able to detect an appreciable amount of the latter mixed with the former. With the exception of the small amount of native erythropoietin found in the asialo preparation, both forms appear to be homogeneous.

Without added surfactant, there is a considerable tendency for native and asialo erythropoietin to aggregate at pH 6 and for the asialo form to aggregate at pH 9, but at pH 7 in the presence of SDS and dithiothreitol, both forms appear to be monomeric. The human asialo hormone has an apparent molecular weight of 34,000 in SDS, whereas the native form has an apparent molecular weight of 39,000.<sup>6</sup> These values contrast with the molecular weight of 41,000 found for sheep plasma asialoerythropoietin by the SDS-gel electrophoretic method and the calculated value of 46,000 for the native form

<sup>6</sup> The molecular weight determined by SDS-gel electrophoresis was the same whether we used 7.5% or 10% gels.

of the sheep hormone (13). When we studied the sheep hormone, it was clear that the Weber and Osborn method of molecular weight determination (11) by gel electrophoresis in SDS was not accurate for the fully sialylated hormone, possibly because of a substantial contribution by the sialic acids to the net charge. At present, we cannot estimate the molecular size of the native human hormone from that of the asialo form since we do not yet have an accurate estimate of the sialic acid content.

The method of iodination of erythropoietin deserves comment. We found with the sheep hormone that the unmodified iodination method (5), in which chloramine-T is used, caused total loss of biological activity. Following the precedent set by Stanley and Metcalf (14), we used a modification of the method of Stagg *et al.* (6) in which the iodination was carried out in 45% dimethylsulfoxide to protect methionine residues from oxidation. In contrast to the findings of Stagg *et al.*, who found no loss of gastrin activity, and those of Stanley and Metcalf who found no loss of colony-stimulating activity, we found that the iodination method does cause appreciable inactivation of erythropoietin. For the case of the preparation with 4 iodine atoms/molecule, it can be calculated that less than 2% of the hormone would be noniodinated. This would suggest that the 25% of the biological activity that remained was due to the iodinated derivative. Until a method can be found for preparation of a fully active, labeled hormone, this less active, labeled erythropoietin may still be useful for the study of a number of

biological characteristics of the hormone which, until now, have not been amenable to experiment.

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